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Novel $P2X_7$ Receptor Antagonists

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Abstract—The synthesis and pharmacological evaluation of a new series of potent $P2X_7$ receptor antagonists is disclosed. The compounds inhibit BzATP-mediated pore formation in THP-1 cells. The distribution of the $P2X_7$ receptor in inflammatory cells, most notably the macrophage, mast cell and lymphocyte, suggests that $P2X_7$ antagonists have a significant role to play in the treatment of inflammatory disease.
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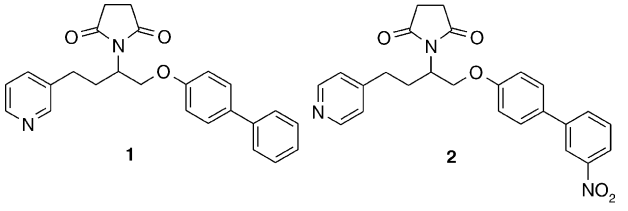
The $P2X_7$ receptor is a ligand-gated ion channel activated by adenosine 5′-triphosphate (ATP). Activation leads to a rapid non-selective influx of cations and, with prolonged exposure to ATP, the opening of large plasma membrane pores.[1] The $P2X_7$ receptor is found on a wide range of inflammatory and immunomodulatory cells, most notably the monocyte/macrophage lineage, mast cells and lymphocytes.[2] Its most notable biological function is the processing and release of interleukin-1β (IL-1β) from monocytes, macrophages and microglial cells, and as such it is implicated in a number of autoimmune and inflammatory diseases.[3] Mice genetically modified to have the $P2X_7$ receptor 'knocked-out' have been reported to show a reduced severity of arthritis in an anti-collagen antibody arthritis model.[4] An antagonist of the $P2X_7$ receptor could therefore provide a new treatment for inflammatory diseases.

Our initial lead compounds came through high-throughput screening of our company compound collection against a functional readout of the $P2X_7$ receptor expressed endogenously on the human pre-monocytic cell line THP-1. The biological screen for the assessment of $P2X_7$ receptor inhibition was the reduction of plasma membrane pore formation induced by a synthetic ATP analogue (benzoylbenzoyl adenosine triphospate-BzATP). This characteristic of $P2X_7$ receptor activation was measured by the entry of a fluorescent DNA probe (ethidium bromide) through the membrane pores with a consequent increase in total cellular fluorescence.[5] The agonist potency order, BzATP > ATP ≥ 2-methylthioATP was also characteristic of the $P2X_7$ receptor. A HTS was undertaken using this assay in 96-well plate format.[6] Estimates of potency of the compounds were made initially from pIC_{50} determinations and then more robustly from pA_2 estimates.

Though the initial hits were weakly active (e.g., **1**,[7] $pA_2 = 5.0$), a clear SAR was observed and it soon became evident that 4-pyridyl was the preferred left-hand side heterocycle and that a 3′-substituent on the biphenyl portion was beneficial for activity (e.g., **2**, $pA_2 = 6.9$).



Very few examples of $P2X_7$ receptor antagonists have been described in the literature.[8] Herein, we report further structure–activity relationship studies carried out around this novel series of antagonist **2** in which replacements for

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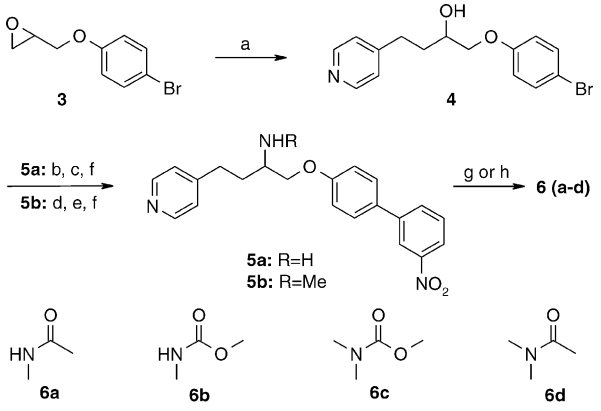
the succinimide and the 4-pyridyl moieties have been investigated.

The synthesis of simple acyclic analogues was carried out as outlined in Scheme 1. Intermediate alcohol **4** was obtained by treatment of epoxyether **3** with lithiated 4-methyl pyridine.

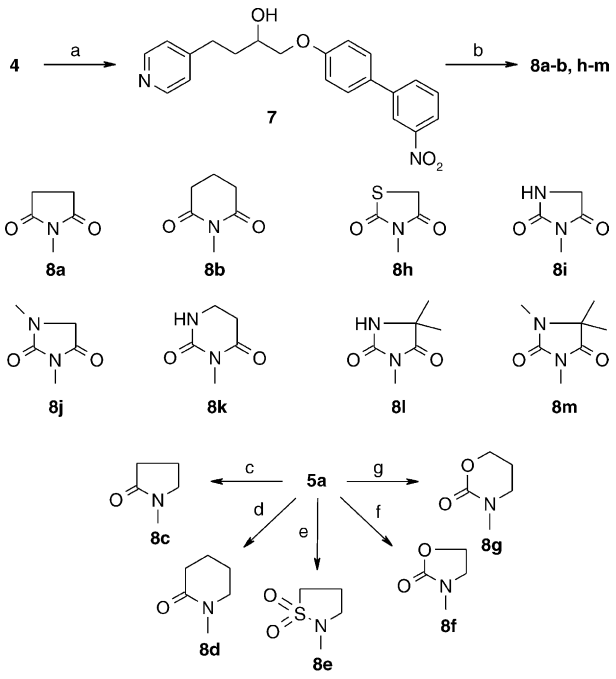


Scheme 1. Reagents and conditions: (a) 4-methylpyrine, *n*-BuLi, −78 °C to rt (64%); (b) phthalimide, Ph_3P, DEAD, THF (98%); (c) 40% aq $MeNH_2$, MeOH, 90 °C; (d) Dess–Martin periodinane, DCM, rt; (e) $MeNH_2$, $NaBH(OAc)_3$, THF, AcOH (60%); (f) (3-nitrophenyl)boronic acid, $Pd(Ph_3P)_4$, Na_2CO_3, EtOH, 80 °C (**5a**: 72%, **5b**: 91%); (g) acetic anhydride, rt (**6a**: 94%, **6d**: 83%); (h) methyl chloroformate, DCM, rt (**6b**: 72%, **6c**: 66%).

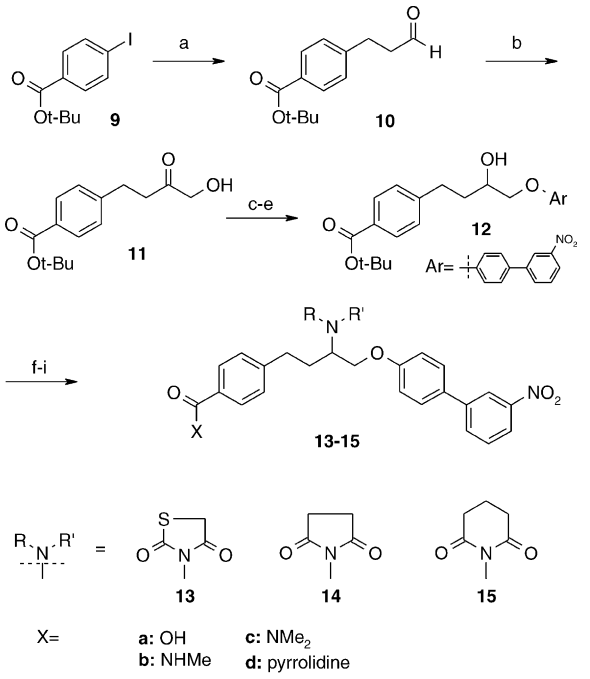
A Mitsonobu reaction using phthalimide then deprotection of the primary amine or, oxidation of the secondary alcohol to the corresponding ketone and reductive amination using methylamine, was followed by a Suzuki coupling with (3-nitrophenyl)boronic acid to yield amines **5a** and **5b**, respectively. The desired acetamides **6a** and **6d** were obtained by reaction of **5a** with acetic anhydride and carbamates **6b** and **6c** by reaction with methylchloroformate.

The preparation of cyclic analogues **8**(**a**–**b** and **h**–**m**) was performed starting from intermediate **4** which underwent Suzuki coupling with (3-nitrophenyl)boronic acid and Mitsunubo reaction using a suitable cyclic nucleophile (Scheme 2). The synthesis of **8c**–**g** was achieved from **5a** by a two-steps-procedure in which the first step was conversion to an acyclic amide, sulfonamide or carbamate bearing a leaving group on the end of the side chain and the second step was a base-mediated intramolecular cyclisation.

4-Substituted benzamides analogues of **8a**, **8b** and **8h** were synthesised as shown in Scheme 3. Aldehyde **10**, derived from a Heck reaction between aryl iodide **9** and allyl alcohol, was converted to the hydroxyketone **11** by means of an acyloin reaction.[9] The primary alcohol was activated as the mesylate and then displaced with 3′-nitrobiphenyl-4-ol. The resulting ketone was reduced to the corresponding alcohol **12**. A Mitsunobu reaction was performed to introduce the cyclic sustituent and the *t*-butyl esters were hydrolysed using formic acid to give acids **13a**, **14a** and **15a**. Finally, the amides **13**, **14** and **15b**–**d** were generated via the acyl chloride.



Scheme 2. Reagents and conditions: (a) (3-nitrophenyl)boronic acid, $Pd(Ph_3P)_4$, Na_2CO_3, PhMe/EtOH, 80 °C (64%); (b) Ph_3P, DEAD, THF (29–65%); (c) 4-chlorobutanoyl chloride, NEt_3, DCM, rt; then *t*-BuOK, THF, rt (44%); (d) 5-chlorovaleryl chloride, NEt_3, DCM, rt; then *t*-BuOK, THF, rt (56%); (e) 3-chloropropane sulfonylchloride, NEt_3, DCM; then EtONa, EtOH, Δ; (f) 2-chloroethyl chloroformate, MeCN, rt, then NaH, THF/DMF, 70 °C (73%); (g) phosgene, PhMe, rt; 3-chloropropanol, rt; then NaH, DMF, 70 °C (33%).



Scheme 3. Reagents and conditions: (a) allyl alcohol, $Pd(OAc)_2$, $NaHCO_3$, $(n\text{-}Bu)_4NBr$, PhMe, Δ (83%); (b) HCHO, cat. *N*-ethyl benzothioazolium bromide, EtOH, Δ (45%); (c) MsCl, NEt_3, DCM, rt (88%); (d) 3′-nitrobiphenyl-4-ol, Cs_2CO_3, MeCN, Δ (57%); (e) $NaBH_4$, MeOH, rt (79%); (f) Ph_3P, DEAD, THF, rt; (g) HOOCH, rt; (h) $(COCl)_2$, DCM; (i) amine, $i\text{-}Pr_2EtN$, DCM, rt.

Initial SAR-studies around **2** focused on an examination of the effects on $P2X_7$ inhibition induced by changes in the cyclic imide portion. As shown in Table 1, simple replacements such as amine **5a**, acyclic amides **6a** and **6d** or acyclic carbamates **6b** and **6c** gave inactive compounds. It would seem that a cyclic substituent is required for potency and the results in Table 1 illustrate tight SARs around the cyclic imide portion of the molecule. Moving from a five- **8a** to a six-membered imide **8b** afforded an increase in activity. Deletion of one of the carbonyl groups dramatically decreased activity with a loss of ~100-fold **8c** and **8d** and the cyclic sufonamide analogue **8e** was poor. Some activity was regained with cyclic carbamates **8f** and **8g**. Consistent with the ring-size effect observed, an increase in potency was seen upon moving to the larger thiazolidine-2,4-dione **8h**. The hydantoin **8i** and, to a lesser extent, the dihydro-uracil **8k** were stable replacements for the imide group, although substitution by methyl groups on the hydantoin moiety abolished all activity **8l–m**. These results demonstrate that this portion of the molecule interacts with a very sterically demanding environment.

Table 1. Inhibiton results for compounds **5a**, **6** and **8**[5]

Compd	Activity pIC_{50}	Compd	Activity pIC_{50}
5a	<5	**8e**	<5
6a	<5	**8f**	6.4[a]
6b	<5	**8g**	6.2
6c	<5	**8h**	7.7[a]
6d	<5	**8i**	6.7
8a	6.9[a]	**8j**	<5
8b	7.5[a]	**8k**	6.2
8c	<5	**8l**	5
8d	5.2	**8m**	<5

[a] pA_2 value.

Even though the succinimide and thiazolidine-2,4-dione groups were suitable substituents for activity, they were metabolically and chemically fragile, being cleaved or hydrolysed readily by nucleophiles. However, even if less active, the carbamates **8f** and **8g** turned out to be more stable metabolically and also more stable chemically to inorganic and organic nucleophiles, having the advantage that it allowed a relatively simple exploration of the biphenyl portion of the molecule through Suzuki coupling on a late stage intermediate and using a parallel approach. Unfortunately, this exercise failed to afford replacements displaying any advantages over the 3-nitrobiphenyl moiety.

Amongst many 4-pyridyl replacements that we explored by rapid parallel synthesis, obvious analogues such as 4-pyrimidine, 4-pyridazine, 4-pyridin-2(1*H*)-one or 4-quinoline failed to yield encouraging levels of activity. However, the 4-substituted benzamide group turned out to be more promising and was studied further. Data for these compounds **13–15** is reported in Table 2. In the thiazolidine-2,4-dione series **13b–d**, the SAR is flat and results in a 10-fold drop of activity compared to the 4-pyridyl. Interestingly, the succimide series **14a–d** showed similar level of potency as the thiazolidine-2,4-dione. Finally, the glutarimides **15a–d** gave the expected increase with respect to the five-membered

Table 2. Inhibition results for compounds **9–11**[5]

Compd	Activity pIC_{50}	Compd	Activity pIC_{50}
13b	6.8	**14d**	6.7
13c	6.8	**15b**	6.9
13d	6.8	**15c**	7.1
14b	6.6	**15d**	7.3
14c	6.6		

imide system with pyrrolidine amide **15b** being of comparable activity to the corresponding 4-pyridyl analogues.

Typical data from the functional screen are shown in Figure 1.

BzATP concentration–effect (E/[A]) curves, 0.3–300 μM, were generated in the absence and presence of compound **8h**, 30, 100 and 300 nM. Increasing concentrations of compound **8h** displaced the location of the BzATP E/[A] to the right progressively, consistent with the action of a competitive antagonist, although there was some depression of the curve with 300 nM of the compound. Clarke analysis of these data gave a pA_2 estimate of 7.7 ± 0.1 ($n = 4$).

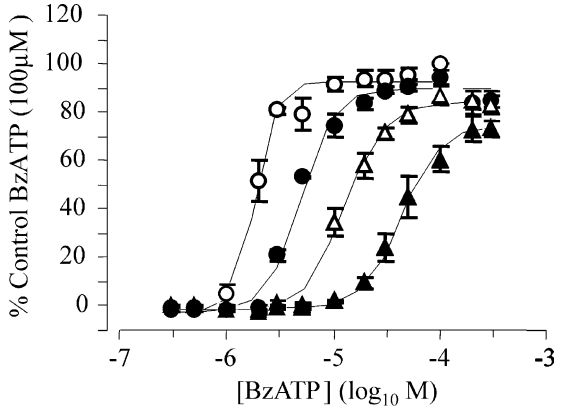


Figure 1. Effects of compound **8h** on the human $P2X_7$ receptor. $P2X_7$ receptor activation was assessed by a fluorescent measurement of BzATP-induced ethidium bromide uptake in THP-1 cells using a standard 96-well-plate fluorimeter. Experiments were performed in a potassium (140 mM), EDTA (1 mM), HEPES (20 mM)-buffered salt solution containing ethidium bromide (0.1 mM) designed to optimize the assay system for $P2X_7$ receptor activation. Symbols represent the following, ○: vehicle control, ●: 30 nM, △: 100 nM and ▲: 300 nM.

We have discovered a novel series of cyclic imides that are potent $P2X_7$ receptor antagonists. SARs around this series show that it is possible to change the metabolically labile thiazolidine-2,4-dione for a stable cyclic carbamate group. We have also found that a 3′-nitro substituent on the biphenyl portion is optimal for activity and that a 4-substituted benzamide group is a suitable replacement for the 4-pyridyl moiety. The accepted link between antagonism of the $P2X_7$ receptor and inhibition of ATP-induced release of IL-1β means that $P2X_7$ antagonists may have a significant role to play in the treatment of inflammatory diseases. Unfortunately, although we were able to find a stable cyclic carbamate replacement for the imide group, the reduction in activity at the $P2X_7$ receptor made this series of compounds less attractive for further exploration.

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5. **Biological materials and methods. General procedures. Cell cultures.** THP-1 cells were grown in roller flask bottles in RPMI 1640 media, containing heat-inactivated foetal bovine serum (10% v/v) and L-glutamine (2 mM), at 37 °C, 5% CO_2, 95% humidity. Cultures were split 1 in 3 when a density of 1.5×10^6 cells/mL was reached (every 3 days). **Plasma membrane pore formation**. BzATP-induced changes in plasma membrane pore formation were assessed by the entry of the DNA-binding dye ethidium bromide (Sigma Chemical Co., Poole, UK). Prior to use in an experiment, cells were transferred to centrifuge tubes and centrifuged at 700*g* for 10 min. The cells were re-suspended at 2.5×10^6 cells/mL in HEPES-buffered salt solution that contained potassium chloride 140 mM, ethylene diamine tetraacetic acid (EDTA) 1 mM, glucose 5 mM, ethidium bromide 0.1 mM and HEPES 20 mM (H 7.4). BzATP (10 μL, pre-diluted in buffer from a 10 mM stock) and either vehicle (10% (v/v) DMSO in HEPES-buffered salt solution, 10 μL) or the appropriate concentration of compound (10 μL in 10% (v/v) DMSO in HEPES-buffered salt solution) was added to the wells of an assay plate (96-well non-sterile white, Costar, Corning, Kent, UK). Cell suspension (80 μL) was then added to each well and the plates were incubated at room temperature for 90 min, after which time the plates were read in a plate fluorimeter (excitation 520 nm; emission 595 nm). For pIC_{50} determinations cells were challenged with 10 μM BzATP (a just-maximal concentration) in the absence and presence of compound and data were expressed as percentage inhibition of the BzATP response. For pA_2 determinations concentration–effect (E/[A]) curves for BzATP were constructed using 1/4-log unit increments (0.3–300 μM) in the presence and absence of compound. Affinity estimates (pA_2) were determined from the relative pA_{50}s of the curves.
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